PROPOSED DESIGN AND INPUT REQUIREMENTS

TITLE:	EXTERNAL RNA STANDARD CONTROLS

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Table of Contents

1. Statement of Purpose

The purpose of this document is to provide specifications that will ultimately guide experimental design, development and testing of external RNA spike-in controls.

Formatting note: Areas highlighted in gray preceding each section include a high level, general statement of user needs and the anticipated utility of each specific feature described. Bullet points indicate common descriptions and specific details.

1.1 Introduction

External standards have been used to validate and interpret data from microarray hybridization experiments since the techniques were first reported in the scientific literature (Fodor 1991, Fodor 1993, Schena 1995, Lockhart 1996). Standards and hybridization controls continue to be valuable tools for assessing the sensitivity, dynamic range, non-specific background and reproducibility of expression array platforms and real-time PCR. To date, however, there are no widely accepted standards that are used across different platforms in the field and no consensus or existing conventions on what those standards should be or how they should perform. A well-characterized set of standards has the potential to set a new benchmark for the field of RNA expression profiling by providing a framework for interpreting, comparing and reporting experimental results, enabling this field to mature and meet its potential.

The External RNA Controls Consortium (ERCC) is composed of representatives from the public, private, and academic sectors, working together to produce external RNA spike-in controls that will be useful for sample control on a variety of microarray platforms and by RT-PCR. As one of many standardization strategies, the ERCC is attempting to establish a set of external RNA spike-in standard controls that researchers can use as exogenous positive controls to monitor assay performance, and also to facilitate comparison of experimental results across platforms.

1.2 Access and usability recommendations

Platform-independent control materials are needed for performance evaluation of reproducibility, sensitivity and robustness in gene expression analysis.

The external RNA spike-in standard controls described in this document are consistent with recommendations made at the 2003 NIST meeting, Metrology and Standards Needs for Gene Expression Technologies: Universal RNA Standards, Cronin *et al.,* 2003. The ERCC will produce recommended protocols for the use of the controls. The ERCC intends for these materials to be readily accessible and have broad acceptance within the research and clinical communities. The controls will consist of 100 well characterized clones comprised of random unique sequence as determined by sequence comparison to mouse, rat, human, drosophila, E. coli, mosquito sequence databases, as well as well characterized Bacillus subtillus and Arabidopsis thaliana clones.

The external RNA spike-in controls will be useful primarily for evaluating sample performance, sensitivity, reproducibility and assay robustness. The ERCC is making every effort to identify unique, specific sequences for the development of the external RNA spike-in controls. However it is impossible to provide absolute assurance of specificity until complete finished genome information is available for every tissue in every developmental stage for every organism. Individuals requiring absolute assurance of specificity will need to test the specificity of the controls in the particular tissues of interest.

The clones, as described above, will be distributed broadly, at minimal charge in a not-for-profit manner. All relevant information regarding the process of sequence selection, clone construction, quality control, characterization, nucleic acid sequences, recommended handling and storage, stability data, recommended protocols for use, quantitative information on nucleic acids, and observed performance characteristics will be published in the open, archival literature and made publicly available.

It is anticipated that commercial development of useful control products will be based upon this library of clones and associated data. Creative adaptation of the library will meet the needs of various applications. Commercial development is expected to include transcript pools created from the library of clones.

Two different pool types have already been planned and are in development, in parallel with the development of the clone library. As described, one set of pools will enable quantitative assessment of dynamic range, and another pool product will permit control of the RNA extraction process.

1.3 References

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2.0 Product Description, Project Phases

The external RNA control reference standards will provide customers with exogenous positive controls to monitor sample performance. This product will be a valuable tool for those customers using microarrays for expression analysis. The controls will also be available in a form useful for RT-PCR.

Product Description

The ERCC proposes to develop two “products” as described in this section:

1) A set of clones appropriate for producing *in vitro* transcripts that will become the external RNA standards.

2) A set of recommendations for using the *in vitro* transcripts as spiked-in external RNA standards.

These recommendations will be validated by the ERCC working group by putting them through the stages of production, quantitation, quality control assessment, cross-reactivity screening and testing on different gene expression platforms. An emphasis will be placed on defining formulations for standardized pools.

Project phases

The goal of the ERCC is the development of a set composed of 96-100 clones appropriate for producing the candidate external standards by in vitro transcription. These clones will be screened for cross reactivity and, if necessary, culled to a set of at least 42-50 clones that will be progressed through further stages of production and testing. This candidate set will represent two functional classes of controls. The first class will be used for the quantitative assessment of sensitivity, dynamic range, transcript abundance and the relative difference in abundance (ratio) between two different RNA samples. The second class of standards will be composed of transcripts of variable lengths for monitoring the fidelity of RNA isolation and labeling methods. This second class may include purified RNA transcripts or encapsulated transcripts. Furthermore, the ERCC will propose a set of DNA sequences for designing capture probes that will serve as negative controls.

The ERCC project falls into two distinct phases. The specific aim of the first phase addresses the definition, assembly and initial production of the clones and transcripts that will serve as candidate exogenous RNA standards. The specific aim of the second phase addresses testing the clones on various platforms and in different pooled configurations. The aim of the second phase is to define an appropriate set of validated transcripts and recommendations for their use on various platforms. This will enable subsequent commercial development and distribution as standardized pooled transcripts for research applications. The completion of the first phase may be a prerequisite to starting the second phase.

Phase 1. Specific Aim: Produce 42-50 clones that meet the following criteria:

1. Standard controls will be produced by *in-vitro* transcription, purified and evaluated for proportion of full-length transcripts as described in the protocols section.

2. Adherence to the guidelines for access and usability outlined in section 1.2.

3. Standards will be assigned membership in one of two classes:
   - Class A standards will be of similar length: 700-800 nt
   - Class B standards will be of variable length ranging from 500 – 2000 nt.
   - The phase 1 goal will be to assemble a minimum of 21 transcripts assigned to each class.

Features of Clones Used to Develop the Standards

Several clone sources have been suggested, including clones originating from intergenic regions of Bacillus subtillis, Arabidopsis thaliana or artificially and randomly derived DNA sequences. It is possible that the source of the clone is less relevant to this project than the performance of the clone, and therefore, the selection of clones shall be based on the following criteria:

1. Adherence to the guidelines for access and usability outlined in section 1.2

2. Minimal predicted cross reactivity with defined transcripts (August 2003) in the genomes of typical research organisms, including, but not limited to the following:
   - H. sapiens
   - M. musculoris
   - S. cereviciae
   - A. thaliana
   - E. coli
   - C. elegans
   - D. melanogoster
   - D. rerio

   Marginal cross reactivity will be defined as having no more than 20 contiguous bases of identity and overall homology less than 70% (For ideas for refining see Hughes 2001).

3. Minimal observed cross reactivity using most major expression microarray formats including chemically synthesized and spotted arrays. Total RNA samples from a variety of cell lines and tissue sources from the list of species above will be labeled and hybridized to microarrays containing oligonucleotide probes from each of the candidate standards. Candidate probes that cross-hybridize with human total RNA samples will be weighted highest, and eliminated from further development. Protocols section 2.3 outlines a method for this procedure.

4. The nucleotide sequence will have a GC content of 40-60% and be void of significant repetitive elements, palindromes or regions of low complexity. Human, mouse and rat genomes are an average of 40-42% GC.

5. The clones will be available to the ERCC by the defined deadline for this phase of the project. *This date is not yet determined.*

6. The inserts will be 0.7 -2 kb in length and cloned in a vector that conforms to the guidelines of section 1.1

7. The vector will contain a poly-A tail of defined length, most likely 30-60 nucleotides. Experiments are underway to evaluate poly-A tail lengths 20-200nt. The vector will contain a T7 promoter for producing the sense stranded transcript. (A standardized vector and E. coli may need to be defined to allow efficient, standardized production of the transcripts and to secure an open path to future development of diagnostic applications requiring FDA approvals.) *What about a poly-A tail and RNA stability in a prokaryote? Is this an issue for production of transcripts in E. coli?*

2.1 Proposed Kit Configurations, Shelf life, Storage

Four distinct standards are envisioned, each a kit of multiple materials to accomplish different components of the control process. Kit 1 will be the artifact produced and distributed by the consortium. Kits 2, 3, and 4 are pools and probes that are expected to be commercially produced and distributed, by consortium members or others. The ERCC will draft specifications, and recommend protocols for development and production of these kits.

Storage:
Store mRNA spike mixtures at –80°C
Store DNA oligonucleotide samples at –15 to –30°C
Store individual clones at –80°C

Kit 1Contents:
Glycerol stocks of clones in an E. coli host strain.

Kit 2Contents:
Pooled transcripts to be spiked into total RNA
- Pool 1 (21 Class A transcripts)
- Pool 2 (21 Class A transcripts)
- Pool 3 (21 Class B transcripts)
- Pool 4 (21 Class B transcripts)

Rationale:
Pools 1 and 2 are partnered for spiking into two different total RNA experimental samples. The intent is to generate a standard curve of standards with equivalent lengths, based on an assumption that this will enable better quantitative assessment of the assay. See the appendix of the protocols section 2.1 for an example of a dilution scheme that provides a spike-in strategy for a standard curve with replicates and defined ratio-metric dosages.

Pools 3 and 4 are a paired set of transcripts similar to pools 1 and 2. The differences are that transcripts are grouped to represent a range of different sizes that are spiked in at low, medium and high dosage levels. This allows assessment of RNA extraction methods, transcript integrity or biases in the detection technique as a function of transcript length.

Kit 3Contents:
Pooled encapsulated transcripts (to be spiked into tissue or cells prior to RNA extraction)
- Pool 1 (21 Class A encapsulated transcripts)
- Pool 2 (21 Class A encapsulated transcripts)
- Pool 3 (21 Class B encapsulated transcripts)
- Pool 4 (21 Class B encapsulated transcripts)

Kit 4 Contents:
Oligonucleotide probe set appropriate for detecting standards on multiple array platforms, including chemically synthesized oligonucleotide and spotted DNA microarray platforms. A plate of 96 amine-modified oligonucleotides will be provided. If we assume the final clone set has 42 members, the plate will have two independent probes for each target standard and 12 negative controls.

2.2 Characteristics of Product Kits

The kit shall work with current sample preparation protocols on most expression array platforms and by RT-PCR. The reference standards will be comprised of 100 component transcripts in prequantitated concentrations. The RNA references standards will be available as individual clones and in pools to allow use in measuring sample quality control at the tissue collection step and at the total RNA step.

- Fragments will be well characterized and source info will be publicly available
- Fragment length shall span 0.70-2 kb,
- Fragments will represent random-generated unique alien sequences, Bacillus subtillus sequences and Arabadopsis sequences
- A detailed description of clones, vector and promoter (designed for independent amplification and keeping in mind intended method of making – either off of plasmid or by PCR)
- A detailed description of set encapsulated or packaged sequences for spike into samples prior to RNA extraction
- Dilution buffers
- 3 quantitated pools, of all transcripts in a range of known concentrations
- 42-50 individual clones, in known concentrations, convenient for dilution, (in first phase)
- Probes and primers for RT-PCR

2.3 Protocols

Protocols will be provided to address the following phases of the project. Emphasis will be placed on procedures for preparing, testing and pooling the transcripts prior to shipping them for use in the various platform-specific labeling procedures.

A. Protocol: Prescreening candidate probes and clones for cross reactivity

Wilson Probes and Clones

50-70mer oligonucleotides will be synthesized that correspond to sequences of the sense strand of the candidate transcripts. The candidate probes selected for microarray printing will be designed according to the principles outlined by Bozdech et al. (Genome Biol. 2003;4(2):R9. Epub 2003 Jan 31.) and specified by the output of the OligoArraySelector software program using the Human genome as the reference data set.

Oligonucleotide probes will be distributed into 384-well polypropylene, U-bottom plates (Genetix, Hampshire, UK) and resuspended at 25μM in 24 μl of print buffer.

Ambion Alien RNA

RNA Sequences are generated using Random DNA Sequence Creator program found at: http://bioinformatics.ccr.buffalo.edu/ToolBox/local/randomdna.html
Sequences are from 700-2000nt in length and range of G+C content from 40-60% including human, mouse, rat, mosquito, Arabadopsis thaliana, E.coli, B.subtillus, etc . Sequences are checked *in silico* for cross hybridization to known genomic sequences. Cloned sequences are generated via assembly PCR using overlapping oligonucleotides. Following DNA sequencing RNA transcripts are synthesized by in vitro transcription.

B. Production and purification of transcripts

This will be detailed in the "Production and QC of ERCC Nucleic Acids" document.

C. Quality control assessment of purified transcripts

RNA integrity will be assessed with the Agilent Bioanalyzer RNA LabChip assay. At least 100ng of each transcript shall be analyzed in duplicate. Using the Bioanalyzer software, the amount of full-length transcript shall be at least 80% within trace. Transcript will be with in 30 nt of the expected size. Stability of the purified transcripts shall be shown by incubating the RNA at 37 C overnight in the storage buffer. This RNA is compared in a Bioananalyzer RNA LabChip assays with identical RNA stored at –20. The two samples (37 vs –20 C) will appear identical when traces are overlaid (i.e. no obvious RNA degradation).

D. Quantitation protocol

The accurate quantification of individual and pooled RNA control spikes is critical for the consistent performance of external RNA standards. The quantification of a pure RNA transcript solution gives the

concentration of the individual species (sequence, molecule, etc.). However, it is only accurate if the purity of the RNA is known and the integrity of the RNA is known to be full-length.

The concentration of RNA stock solutions and final pooled mixtures will be determined by measuring the absorbance at 260 nm using well accepted nucleic acid principles and practices. Use of a NanoDrop Spectrophotometer can reduce the possibility of errors created while making calculating dilutions, as this instrument can measure A 260 nm between 0.2-50.

Spectrophotometer: calibrated and validated instrument
Cuvettes: High quality quartz cuvettes (cleaned and scratch free) greater than 100 ul volume.
Measurement values: Performed in triplicate between 0.2-1.0 A
Buffer for dilutions should be low salt and pH 7.0 (10 mM Tris HCl/ 0.1mM EDTA)
Dilution volumes: No dilution volumes under 10 ul or greater than 1 ml.
Dilution vessels: Non-stick microcentrifuge tubes are preferred (no glass).
Conversion Factor: 1A at 260 nm = 40 ug/ml using 1 cm path length. The molecular weight = 330 x length of RNA (nt).

Carrier RNA should not be used in dilution buffers for measuring RNA concentration.

Example Calculation:
-Stock RNA is 100ul.
-A 1:50 dilutions is made (10 ul of RNA mixed with 490 ul TE).
-A260 reading taken in spectrophotometer. A260 = 0.42.
-Concentration is equal to 40 ug/ml x 0.42 x 50 = 840 ug/ml RNA in stock solution
-Total yield is equal to 840 ug/ml x 0.1 ml = 84 ugs RNA in stock solution

E. Dilution protocol

Stock solutions
Individual transcript stocks will be dissolved at 100 ng/_l in stabilization buffer 1 mM Na Citrate pH 6.4 or 0.1 mM EDTA pH 7.0).

Working solutions for PolyA RNA External Controls Carrier RNA. Dilutions should be made in 10 mM TrisHCl 0.1 mM EDTA pH 7.5 with 10ng/ul Yeast tRNA.

Further dilution of the stocks will be performed to create labeling pools as described in the next section.

F. Pooling protocols

Protocols will be provided as examples until testing can validate the utility of each. They will likely require adjustment to the formulations after initial testing results have been generated. The production will also need to be scaled up appropriately once a set of pooling formulations has been adopted.

See Appendix 1 for example dilution protocol.

G. Storage protocol

Storage Temperature- All RNA/DNA materials shall be stored at –80o C for “Long term storage” and –20oC for typical use.
RNA Storage buffer- RNA should be stored in 1 mM Na Citrate pH 6.4 or 0.1 mM EDTA pH 7.0.
RNA carrier- Yeast tRNA (or equivalent) should be used at concentration of 10 ng/ul to aid in the stabilization of RNA solutions for long term storage and for making working solutions.
Shelf life- RNA should be stored no longer than 15 months from the date of manufacturing if properly stored.
Storage Concentrations- Stock dilutions of individual or pooled transcripts will be stored at -80°C, dispensed in at least 20 microliter aliquots between 1 – 0.1 micrograms/microliter in storage buffer.

H. Shipping protocol

RNA and DNA materials shall be shipped by reputable courier (FedEx, UPS, etc.) using overnight delivery between Monday and Thursday. RNA and DNA materials will be placed in appropriate sized plastic screw-top tubes, inserted into small cardboard box and sealed in plastic bag. Entire package will be placed in a Styrofoam cooler and covered with at least 3 lbs (1.5 kg) of dry ice. At no time should the

RNA/DNA material be thawed during this process. If material arrives with dry ice than it can be assumed intact. If no dry ice is present upon receipt the RNA should be discarded.

I. Protocols for use

Addition of pools to total RNA for labeling and cleanup of targets

- cDNA labeling in the context of total RNA from eukaryotes
  - by direct incorporation of dUTP-fluor
  - example see "NIAID Labeling and Hybridization Protocol V2" as an appendix.
  - by incorporation of amino allyl and coupling to fluorophores
- cDNA labeling in the context of total RNA from prokaryotes
  - by direct incorporation of dUTP-fluor
  - by incorporation of amino allyl and coupling to fluorophores
- cRNA labeling in the context of total RNA from eukaryotes
  - incorporation of biotin and detection with phycoerythrin
  - incorporation of amino allyl and coupling to fluorophores
  - incorporation of fluorescent nucleotides
- Hybridization, washing, staining, scanning protocols
  -

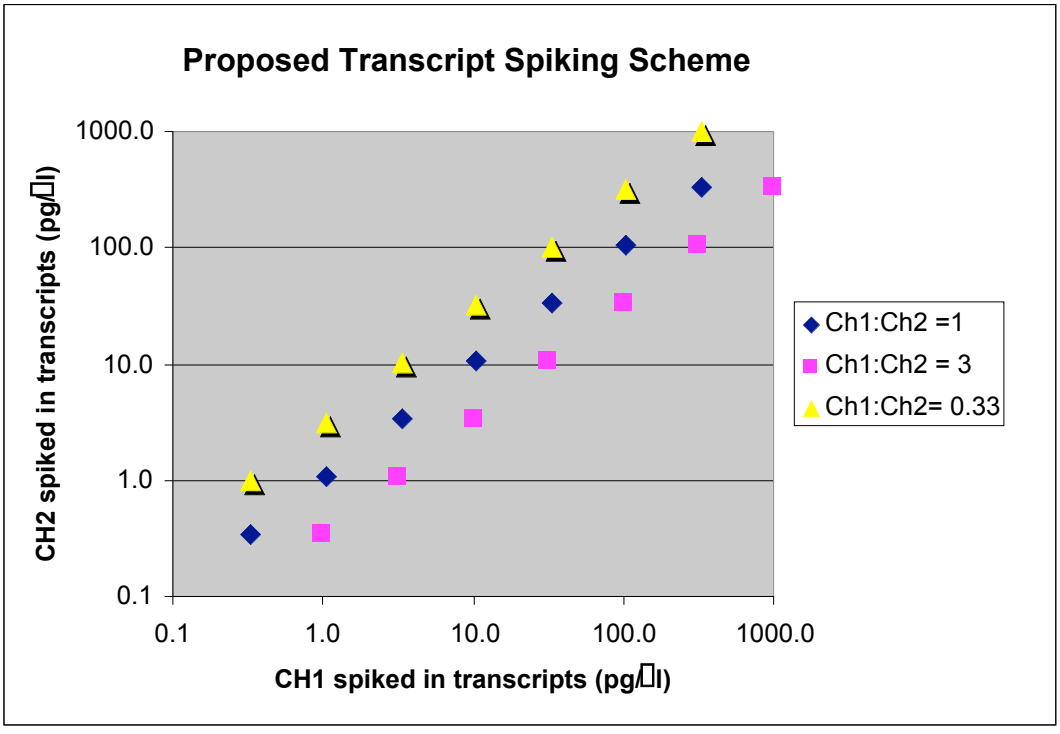
J. Encapsulated pool protocol for use

Armored RNA Spikes- http://www.ambiondiagnostics.com/products/armored_rna.html

K. Appendix of Example Protocols

Example 1. Standard curve with 3-fold input differences between two samples.

A proposed pooling protocol will consider the situation where two different transcript pools are created for 21 external control transcripts. The dilution series covers 3-logs of dynamic range using a 3.16-fold dilution series. (This dilution series was chosen to create a linear log-log plot with equal spacing between points as shown.)



Key features:

- Triplicate representation of each of the 7 spike-in doses for CH1, covers a 3-log range.
- Triplicate representation for 5 of the spike-in doses for CH2, covers a 4-log range

- CH1 and CH2 presume the two-color hybridization mode. The same scheme can be applied to compare two samples in two separate hybridizations in the single-channel mode or RT-PCR applications.
- Sensitivity is defined by the lowest detectable signal which is greater than the 95% confidence interval provided by the set of negative control probes on the microarray.
- Accuracy of ratio measurement will be assessed for each transcript based on the deviation from expected ratio.

Dilution and pooling procedure for Example 1.

Seven different transcripts will be pooled into each of three separate groups: A, B and C. These pooles will then be combined into two different master pools for spiking into two different labeling reactions. This scheme produces defined ratios for each transcript (1:3, 1:1, 3:1). To achieve the appropriate dynamic range, the seven transcripts will be combined using a 2-stage dilution process that requires the formation of a prepool of three transcripts for each group as shown in Table 1.

Reagents:
Transcript stocks will be dissolved in stabilization buffer to a concentration of 100 ng/ml.
Yeast tRNA, RNase-free (100ng/ml)
TE Buffer: 10mM Tris, pH 7.4, 1 mM EDTA

Dilution and pooling procedure:
Step 1: Thaw stocks on ice.
Step 2: Label 3 tube “prepool A”, “prepool B” and “prepool C”
Step 3: Keeping the tubes on ice, add the volumes and reagents shown in table 1.
Step 4: Mix the contents by vortexing and centrifuge briefly to push the liquid to the bottom of the tube.

Table 1: Formulation of prepools.

Prepool A		Prepool B		Prepool C	
Transcript ID	Volume (µl)	Transcript ID	Volume (µl)	Transcript ID	Volume (µl)
tRNA	2.00	tRNA	2.00	tRNA	2.00
TE	5.44	TE	5.44	TE	5.44
Tx01	1.00	Tx08	1.00	Tx15	1.00
Tx02	3.16	Tx09	3.16	Tx16	3.16
Tx03	10.00	Tx10	10.00	Tx17	10.00
total	20.00	total	20.00	total	20.00

Step 5: Label 3 tube “Pool A”, “Pool B” and “Pool C”
Step 6: Keeping the tubes on ice, add the volumes and reagents shown in Table 2.
Step 7: Mix the contents by vortexing and centrifuge briefly to push the liquid to the bottom of the tube.

Table 2. Formulation of Pools A, B and C prior to creation of the master pools.

Pool A		Pool B		Pool C	
Transcript ID	Volume (µl)	Transcript ID	Volume (µl)	Transcript ID	Volume (µl)
tRNA	20.00	tRNA	20.00	tRNA	20.00
TE	33.24	TE	33.24	TE	33.24
Prepool A	2.00	Prepool B	2.00	Prepool C	2.00
Tx04	3.16	Tx11	3.16	Tx18	3.16
Tx05	10.00	Tx12	10.00	Tx19	10.00
Tx06	31.60	Tx13	31.60	Tx20	31.60
Tx07	100.00	Tx14	100.00	Tx21	100.00
total	200.00	total	200.00	total	200.00

Combining Pools A, B and C according to the following table to achieve defined proportions
Step 8: Label 3 tube “Master Ch1”, and “Master Ch2”
Step 9: Keep the tubes on ice, combine the contents of Pools A, B, and C as shown in Table 3.
Step 10: Mix the contents by vortexing and centrifuge briefly to push the liquid to the bottom of the tube.

Table 3. Creation of master pools for spiking into sample RNA

	Ch1 volume (µl)	Ch1 proportion	Ch2 volume (µl)	Ch2 proportion
Pool A	39	0.2	117	0.6
Pool B	39	0.2	39	0.2
Pool C	117	0.6	39	0.2
total	195	1.0	195	1.0

The concentrations of each transcript is shown in Table 4.

Table 4. Concentrations and input ratios of each transcript in the pools

Transcript ID	Ch1 master pool concentrations (pg/µl)	Ch2 master pool concentrations (pg/µl)	Ch1:Ch2
Tx01	10	30	0.33
Tx02	32	95	0.33
Tx03	100	301	0.33
Tx04	317	951	0.33
Tx05	1001	3004	0.33
Tx06	3165	9494	0.33
Tx07	10000	30000	0.33
Tx08	10	10	1
Tx09	32	32	1
Tx10	100	100	1
Tx11	317	317	1
Tx12	1001	1001	1
Tx13	3165	3165	1
Tx14	10000	10000	1
Tx15	30	10	3
Tx16	95	32	3
Tx17	301	100	3
Tx18	951	317	3
Tx19	3004	1001	3
Tx20	9494	3165	3
Tx21	30000	10000	3

Notes on pooling example 1:
As shown in this example, the master pools for Ch1 and Ch2 are approximately 100-fold more concentated than would be used in the actual labeling reaction. We assume that the spikes will be put into the labeling reactions as part of a master mix of labeling reagents and enzymes for processing multiple samples.

An Excel spreadsheet was created for the design of this protocol that allows the user to change the key assumptions about the concentrations and reagents. It should be useful in further optimization of this method and it is available to ERCC members upon request from mwilson@niaid.nih.gov

2.3 Testing

Testing will be performed to evaluate the performance of candidate transcripts across platforms. Based on this information, the final set of transcripts will be selected for use as the external RNA spike-in standard controls. Pooled standard controls will be developed from the selected set.

Transcripts shall be evaluated on the following three platforms:

- **One-Color Microarrays** – Biotinylated targets from a single RNA sample hybridized to one microarray that contains multiple probes for each transcript (e.g. Affymetrix).
- **Two-Color Microarrays** – Cy3- and Cy5-labeled targets generated from two RNA samples and simultaneously hybridized to one microarray that contains a single probe for each transcript (e.g. Agilent).
- **Quantitative, Real-Time PCR (qRT-PCR)**

(Additional platforms to be determined.)

During testing, each transcript will be evaluated for consistent, accurate within-sample values (for sample QC) and between-sample values, such as Fold Change (for platform comparisons). RNA standard testing shall be conducted in consecutive phases:

Phase I – Two RNA pools that contain all candidate transcripts will be compared. For each individual transcript, one pool will contain the standard at a low concentration, while the other pool will contain the standard at a high concentration. The test will be replicated in order to evaluate reproducibility and the ratio of the expression values in each pool will be compared to the expected fold change. Transcripts with poor performance will be eliminated from the later phases of testing.

Phase II – A series of RNA pools that contain different dilutions in a Latin Squares design will be used to develop a standard curve for each transcript. The test will evaluate the specificity and sensitivity of each transcript.

Phase III – If necessary, a third phase of testing will confirm that the final pools of external RNA standards perform well in multiple laboratories under a variety of conditions.

Reagents

RNA Standards

Initially a set of 42-50 candidate transcripts of different lengths, representing alien, Bacillus and Arabidopsis sequences will be tested. The concentration and quality of each transcript will be verified using a spectrophotometer and Agilent BioAnalyzer, respectively.

The transcripts will be pooled (as described below) and distributed to the testing laboratories. The quality of each pool will be confirmed before and after shipment using an Agilent BioAnalyzer.

Microarray Design

We suggest that the microarrays used in the RNA standard testing contain the following features:

- The arrays must contain oligonucleotides complementary to each of the RNA standards. The two-color arrays will contain at least one oligonucleotide probe per transcript, while the one-color arrays will contain at least one probe set for each transcript.
- The sequences of the oligonucleotide probes will be selected by the manufacturers according to their standard protocols.
- If possible, the arrays should contain more than one probe or more than one probe set for each transcript. With this design, the quality of the oligonucleotides can be evaluated during the RNA standard testing and the more effective, uniform probes or probe sets can be selected.
- The arrays shall contain probes or probe sets from both the 5' and 3' regions of the transcript for not less than ten of the RNA standard controls.
- The arrays may contain replicate spots of the same probe or probe set, in order to determine the intra-array variability.
- The external RNA transcripts will be spiked into a complex RNA background during testing. The arrays should contain probes or probe sets for genes expressed in the complex background (e.g. human beta-actin). These probes might detect bias in the RNA standards system or could be used for normalization. An ideal array design would be a standard human catalog design, modified to contain the additional probes needed to detect the spike-in targets.

qRT-PCR Primer Design

The primers used in qRT-PCR shall include the following features:

- Two sets of primers for each transcript to evaluate both the 5' and 3' ends of the transcript.
- Amplicon lengths between 100 and 200 bp, preferentially below 150 bp., enabling the use of the same set of primers in either SYBR Green or probe-based detection systems.
- For Arabidopsis transcripts, at least one of the primers in each set will span an intron/exon junction, so that contaminating DNA will not be amplified [Note: this will not be possible for Bacillus or alien sequences].
- The primers will be optimized for concentration and ratio (forward to reverse) with Ct (threshold cycle) as the first criterion and final fluorescence as the second criterion. This optimization step will identify any fluorescent signal from primer-dimers or non-specific amplicons.

Experimental Design Examples

This section describes potential experimental designs for each platform that could be used during two phases of testing. Representative tables are provided to suggest how the data could be presented and evaluated. These tables do not include rows for each candidate transcript.

Phase I Testing

Phase I testing provides a quick screen of the performance of each transcript and its ability to detect known expression differences in two complex RNA mixtures. It requires two pools of RNA, referred to as A and B) that contain all of the candidate transcripts at either a high or low concentration. For simplicity, all transcripts will be evaluated at the same two concentrations labeled high and low, generating the same expression ratio. However, some transcripts will be present at the high concentration in pool A and others will be present at the high concentration in pool B. This design ensures that each pool has equivalent amounts of total RNA.

	Level in Pool A (copies/cell)	Level in Pool B (copies/cell)	Expected A/B Ratio
Transcript 1	[Low]	[High]	
Transcript 2	[High]	[Low]	
Transcript 3	[Low]	[High]	
…			
Transcript 50	[High]	[Low]	

One-Color Arrays

1. Spike pool A into human Universal Reference RNA from Stratagene.
2. Spike pool B into human Universal Reference RNA from Stratagene.
3. Prepare four targets for each of the spiked RNA samples (8 total).
4. Hybridize each target to a single Affymetrix array (8 total).
5. Extract Signal values and scale to an appropriate target intensity.
6. Log transform and average replicates.
7. Determine the average Log(A) to the average Log (B) ratio.

Signal is the background-subtracted, normalized expression value using Affymetrix software.
Ave Log(A) represents the mean value for the four log-transformed A array Signals.

Probe	Pool A RNA					Pool B RNA					Ave.Log(A) to Ave.Log(B) Ratio
	Array A1 Signal	Array A2 Signal	Array A3 Signal	Array A4 Signal	Ave. Log (A)	Array B1 Signal	Array B2 Signal	Array B3 Signal	Array B4 Signal	Ave. Log (B)	
Transcript 1											
Transcript 2											
Transcript 3											
…											
Transcript 50											

Two-Color Arrays

1. Spike pool A into human Universal Reference RNA from Stratagene.
2. Spike pool B into human Universal Reference RNA from Stratagene.
3. Prepare two Cy3-labeled targets for each of the spiked RNA samples (4 total).
4. Prepare two Cy5-labeled targets for each of the spiked RNA samples (4 total).
5. Hybridize two Agilent arrays with a 50:50 mixture of the Cy3-A target and the Cy5-B target (referred to as Array 1 and Array 2).
6. Hybridize two Agilent arrays with a 50:50 mixture of the Cy3-B target and the Cy5-A target (referred to as Array R1 and Array R2)
7. Extract Cy5/Cy3 ratios for Array 1 and Array 2.
8. Extract Cy3/Cy5 ratios for Array R1 and Array R2.
9. Log transform and average the Log(A/B) values

Probe	Pool A-Cy5 Pool B-Cy3		Pool A-Cy3 Pool B-Cy5		Ave. Log(A/B ratio)
	Array 1 A/B Ratio	Array 2 A/B Ratio	Array R1 A/B Ratio	Array R2 A/B Ratio	
Transcript 1					
Transcript 2					
Transcript 3					
…					
Transcript 50					

A/B ratio is the background-subtracted, normalized Cy3/Cy5 or Cy5/Cy3 intensity ratio.
Ave. Log(A/B) represents the mean value for the four log-transformed A/B ratios.

qRT - PCR

- Select one transcript to be the "normalizer".
- Determine the concentration and copy number of the normalizer transcript.
- Design and optimize two sets of primers for each transcript (5' and 3').
- Spike pool A into human Universal Reference RNA from Stratagene.
- Spike pool B into human Universal Reference RNA from Stratagene.
- With each RNA pool, run duplicate SYBR Green-based, one-tube kinetic RT-PCR reactions using rTth DNA polymerase (or equivalent), an ABI Prism SDS 7900HT instrument (or similar platform) and two primer sets for each transcript.
- Extract Ct values.
- Average replicates and calculate delta Ct.
- Determine copy number for each transcript by comparing to the normalizer transcript.
- Compare copy number in pool A to copy number in pool B.

Primers	Pool A RNA					Pool B RNA					PoolA to PoolB Ratio
	RT-PCR A1 Ct	RT-PCR A2 Ct	Average Ct	Delta Ct	Copy Number in Pool A	RT-PCR B1 Ct	RT-PCR B2 Ct	Average Ct	Delta Ct	Copy Number in Pool B	
Normalizer Transcript				--					--		
5' set Transcript 1											
3' set Transcript 1											
…											
5' set Transcript 50											
3' set Transcript 50											

Phase II Testing

Phase II testing generates a standard curve for each transcript. For microarray experiments, the transcripts will be evaluated together in a series of pools in Latin Squares or Graeco-Latin Squares design. The transcript concentrations in the pools will be chosen to span a concentration range from 0.1 copies/cell to 1000 copies per cell and an expression ratio range from 0.01 to 100. (A less rigorous methodology for creating standard curves may be employed if resources are not identified to run full Latin or Greco-Latin designs.)

One-Color Latin Square

		Arrays			
		A_1	A_2	A_3	A_4
Transcripts	T_1	L_1	L_2	L_3	L_4
	T_2	L_2	L_1	L_4	L_3
	T_3	L_3	L_4	L_1	L_2
	T_4	L_4	L_3	L_2	L_1

Two-Color Graeco-Latin Square

		Arrays			
		A_1	A_2	A_3	A_4
Transcripts	T_1	gL_1, rL_1	gL_2, rL_2	gL_3, rL_3	gL_4, rL_4
	T_2	gL_2, rL_4	gL_1, rL_3	gL_4, rL_2	gL_3, rL_1
	T_3	gL_3, rL_2	gL_4, rL_1	gL_1, rL_4	gL_2, rL_3
	T_4	gL_4, rL_3	gL_3, rL_4	gL_2, rL_1	gL_1, rL_2

L is the concentration of the transcript in the RNA pool used to prepare the biotinylated target for a one-color hybridization. gL and rL represent the concentrations of the transcripts in the RNA pools used to prepare the green Cy3 target and the red Cy5 target, respectively, for a two-color hybridization.

For qRT-PCR experiments, the transcripts will be evaluated individually. Each transcript will be diluted to a series of concentrations and run in duplicate. R-square and slope values will be extracted from each standard curve and used to determine PCR efficiencies.

Analysis

Both within-sample data (for sample QC) and between-sample data (for platform comparisons) will be evaluated. The performance of each transcript shall be evaluated based on the following criteria:

- Reproducibility - The testing proposal includes 2-4 replicates for each platform, which can be used to determine reproducibility. Possible reproducibility measures could include: Ct (qRT-PCR), probe hybridization intensity (one and two-color arrays, both channels), transcript detection (one and two-color arrays) and probe set Signal value (one-color arrays).
- Sensitivity – Standard curves generated during phase II testing will determine the dynamic range and sensitivity of each transcript.
- Specificity – Specific targets will be missing from some of the pools during phase II testing. This design will help determine the specificity of the transcripts and oligonucleotide probes.
- Fold Change – Between-pool comparisons will evaluate the ability to detect known concentration differences between pools.

Each transcript will be evaluated for its performance on individual platforms and across all platforms. The exact platform-specific and across-platform specifications will be outlined in section 2.1.6.

2.5 Definitions and Assumptions for Measuring Acceptable Performance

The goal is to develop a set of RNA standards that will provide a range of signal intensities and/or gene expression ratios in an array that roughly mimics what can be found in "normal" mammalian cells. For other organisms (plants, birds, etc.), the amounts and types of sequences will have to be adjusted accordingly.

- An average mammalian mRNA is ~1.7kb, M.W.=1,700nt x 330g/mol.nt = 5.6×10^5 g/mol
- The amount of RNA per mammalian cell varies somewhat, but is approximately 26pg RNA/cell (**Mol Biol of the Cell Chapt 8, New York and London: Garland Publishing, 1994, pages).**
- The mRNA/Total RNA ratio varies considerably by cell or tissue. Example data (Qiagen Oligotex Handbook, May 2002, 2nd Edition) are lower limits estimated by isolation of poly-adenylated mRNA from purified total RNA samples.

Tissue	mRNA % of Total RNA*	Total RNA	mRNA
Brain	4.2%	120ug	5ug
Liver	3.5%	400ug	14ug
Kidney	2.6%	350ug	9ug
Intestine	1.3%	150ug	2ug
HeLa 10^7 cells	2.0%	150ug	3ug

* estimated lower limits

- Given the variance in RNA content and mRNA/Total RNA ratio by tissue, it is suggested that sensitivity be defined in terms of mRNA per Total RNA abundance. Hence sensitivity will be expressed as molecules mRNA for a given gene per 100,000 mRNA molecules, or as parts per million, rather than "copies per cell".
- Similarly, "RNA sensitivity" will be defined in parts per million, independent of the ug RNA starting material.
- We assume a polydT primer is used to label the mRNA fraction of total RNA. If random priming or purified polyA RNA are used, the calculation can be simplified accordingly.

Example: Suppose one wishes to test sensitivity in 1 part per 100,000. What quanitiy and concentration of spike-in is required?

- # moles spike RNA =(#parts per million desired)(moles mRNA in #ug total RNA) or:
- # moles spike RNA = (1/100,000) (#ug total RNA)(1g/10^6ug)(%mRNA/100%)(1mole mRNA/560,000g/mole)
- e.g.: # moles spike RNA = (1/100,000) (20ug total RNA)(1g/10^6ug)(3.5% mRNA/100%)(1mole mRNA/560,000g/mole) or:
- # moles spike RNA = 1.25 x 10^{-17} moles

The following generalizations will be useful to determine the approximate ranges for RNA spikes. An "average" cell contains ~26 pg total RNA and ~2% of the total RNA is mRNA. The average molecular weight of an mRNA species is ~560,000 gm/mole **(reference ?)**. If using 20 ug total RNA for labeling, then this is equivalent to isolating RNA from 1 million cells. If one wishes to set as a lower limit the detection of 1 copy of mRNA per cell, then 20 ug will contain 1 million copies of the rare mRNA species. One million molecules is approximately 2000 femtomoles or 2 X 10^{-18} moles, and this is ~1 pg of mRNA.
Calculations: 1 X 10^6 molecules / 6 X 10^{23} molec/mole = 1.7 X 10^{-18} moles
(5 X 10^5 gm/mole)(1.7 X 10^{-18} moles) = 8.5 X 10^{-13} gms mRNA

Testing Performance

To test the concentration of mRNAs from rare to abundant, a range from 1 copy per cell to 10,000 copies per cell seems reasonable, as 10,000 copies should max out most array scans. Thus, a dilution series of RNA "spikes" could contain a relatively small number of concentrations such as:

SPIKES: 0, 1, 10, 50, 100, 200, 500, 1000, 2000, 5000, 10000 copies

For ratio-metric studies, a similar small number should also suffice. The intensity of the 1 part of the ratio should be about 5X background or in the solidly significant signal range.

RATIOS: 50/1, 25/1, 10/1, 5/1, 2/1, 1/1

For the intensity spikes, one would need only 10 different sequences and for the ratio spikes, 12 would be required. If a few negative controls are desired, then the total number of RNA standards can be 25-30 or less. Each control would be represented in quadruplicate or more on the slides so that good statistical measurements can be made for each. The control spots should also be spread somewhat "randomly" throughout the slides for better "sampling" of the intensity data.

Performance Requirements

What is the lowest signal (lowest copy number) one should reasonably detect from array to array?

What dynamic range of performance will the control pools be designed to address?

Open for discussion.

2.6 External RNA Control Quality Control by Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

External RNA standard control quality and concentrations will be evaluated using qRT-PCR.

RT-PCR reagents will be developed to enable performance evaluation of the standard controls by a second well-characterized and controlled methodology. Protocols will be developed to enable performance comparison by a number of different RT-PCR methodologies.

Background/Introduction

The external RNA standard controls will be generated by *in vitro* transcription from plasmid DNA templates, and their quality, concentrations and other properties will be characterized by qRT-PCR. Gel electrophoresis will be used to examine the entirety, sizes and purity of RNA transcripts, but is not sufficient for determining the potential cross reactivity of RNA transcripts nor the trace amounts of DNA templates that might contaminate the RNA preparations. Absorbance at 260 nm of isolated purified nucleic acids is often used to determine physical concentrations of RNA preparations. However, for many biological experimental protocols or *in vitro* diagnostics, it is not possible to measure the concentrations of very limited starting materials, or a specific species of RNA or DNA within a nucleic acid extraction. Thus, it is desirable to have reference methods for quality control assessments, particularly for determination of the relative concentrations of specific transcripts within complex mixtures. For this purpose the relationship between pure physical concentrations of a given RNA control reference and its relative concentrations needs to be measured quantitatively by one or more "Gold Standard" assays. Furthermore, for use in establishing performance of complex systems like multiplexed RT-PCR or microarrays, many different RNA control transcripts are required in pools of varying relative and absolute concentrations in order to mimic the many mRNA species that might be contained in a given RNA preparation from a biological sample. For such assays, properties such as sensitivity, specificity, linearity, and other characteristics need to be established. Finally, experimental results using standardized external RNA controls using different technology platforms (e.g. microarray, RT-PCR) will need to be compared.

In the following work plan, quantitative RT-PCR is used for quality control testing of two external RNA control pools to be prepared. RT-PCR will be used to determine the relative concentrations of a subset of external RNA controls within each pool and will be compared with the physical concentration assessed by UV spectroscopy in pure form prior to dilution. Furthermore, in addition to descriptive assessment of individual transcripts by Agilent Bioanalyzer electrophoresis, the degree of full length transcript prepared will be assessed using RT-PCR primer pairs targeting both the 3' and 5' ends of the synthetic transcripts. This will allow assessment of assay characteristics in reporting on full length transcript abundance when a part of the mixed external RNA control pools as well as when spiked into total RNA preparations, or as encapsulated spike controls for biological samples. Finally, alternative RT-PCR protocols including technical information relevant for comparability of different RT-PCR methodologies will be described. The properties and characteristics of external RNA controls determined by RT-PCR QC procedures will allow comparison microarray results.

Work Plan

1. Determination of physical properties of external RNA controls

A subset of external RNA control transcripts and their encapsulated forms will be provided by the ERCC. The entirety and homogeneity of the pure RNA transcripts will be examined by denatured RNA gel electrophoresis as well as by the Agilent Bioanalyzer 2100 instrument. In addition, absorbance of the RNA transcripts at A260/280 will be measured to provide information about the concentration and purity of synthetic RNA transcripts. With sequence information for each transcript provided, the numbers of RNA molecules in a given preparation can be estimated to serve as the basis for determination of relative concentrations of those RNA transcripts in pools. For comparison, the concentrations of RNA transcripts can also be measured by the RiboGreen assay (Molecular Probe).

For UV absorbance measurements of encapsulated external RNA controls they will first be purified to remove all proteins. There may be some discrepancies between the amounts of initial external RNA controls encapsulated and those purified by protein extraction due to loss during the purification steps.

2. Design of RT-PCR primers for external RNA controls

RT-PCR primers will be designed for the subset of external RNA control transcripts. For each RNA transcript, at least of two sets of RT-PCR primers will be designed by Oligo 6, Vector NTI, or other standard primer design programs. One of RT-PCR primer set will be located at the 3' end of the RNA transcripts, while another will be positioned at the 5' end. The lengths of specific primers designed will be between 18-25 nucleotides in length and the size of amplicon generated designed to be < 200 bp. Primer design considerations will be given to allow these amplicons to be used for both SYBR-based and Taqman-based RT-PCR. In addition, the Tm for primers and the sizes of amplicons for both 3' and 5' ends will be designed to be as similar as possible to minimize amplification efficiency differences between to two RT-PCR primer sets used per transcript.

3. Quality Control assessment of external RNA controls

Two sets of RT-PCR primers at 3' and 5' ends will be used to determine the entirety and specificity of RNA transcripts. The ratio of 3' to 5' RT-PCR quantitation results should be approximately 1. Significant departure from this ideal ratio of 1 may be indicative that a proportion of the RNA transcripts are not full-length. This quality control step is particularly useful for the encapsulated RNA controls. In addition, by subjecting the external RNA control transcripts to PCR reactions with or without a RT step, information will be provided regarding DNA template contamination of the RNA preparations.

Stability of external RNA control transcripts will also be determined by RT-PCR. Once the relative concentrations of external RNA controls have been determined (see below), the stability of external RNA controls can be monitored under different storage or shipping conditions.

4. Determination of relative concentrations of external RNA controls by RT-PCR

For each pool one pure external RNA control transcripts will be chosen as for determination of relative concentrations of other transcripts ("copy numbers") within pools using RT-PCR protocols. The relative concentration of one transcript will be compared with its physical concentrations as well as with its concentration in the encapsulated form. Using this RNA transcript as a standard, the relative concentrations for other external RNA control transcripts and their encapsulated forms will be determined.

Copy numbers and the relative concentration of external RNA controls will be determined using an "end-point" limiting dilution assay. The RNA transcript will first be diluted to about 1 copy per PCR reaction. A large number of RT-PCR reactions (about 500) will be performed using this concentration of the RNA transcript. Then, the copy number per PCR can be calculated by using the equation of "Copy number (RT-PCR signal generating unit)/PCR = -ln (numbers of negative PCR reactions/total numbers of PCR reactions)" followed by the statistical analysis of a Poisson distribution. To ensure the accuracy of the determined copy numbers, RT-PCR reactions using two sets of PCR primers against the same RNA transcript at 3' and 5' ends will be performed for determining its relative concentration.

The "end-point" limiting dilution assay has been successfully used in determining copy numbers of internal RNA controls for many viral load assays. However, these assays are highly dependant on assay conditions including purity of RNA transcripts, the specific PCR primers and polymerases used, the sizes and locations of amplicons produced, the specific instruments and PCR conditions employed, etc. In order to standardize all RT-PCR reagents and instruments for copy number determination we propose to use Roche's reagents (available through Applied Biosystems) and ABI instruments for now. After determining copy numbers of the first control RNA transcript, it can then be used as a reference to determine copy numbers of the rest of external control RNA transcripts.

In order to use this RNA transcript as a reference, we also need to know the detection limit and quantification range of the assay. A serial dilution of the RNA transcript will be made based on its physical concentration and its relative concentration. By performing enough numbers of PCR reactions at different concentration levels, the dynamic range of the assay and limit of transcript detection can be readily assessed. With this RNA control standard, the relative concentrations of other external RNA controls can be quantitatively measured.

5. Characteristics of pooled external RNA controls and encapsulated preparations

To different mRNA species with different expression levels in a given total RNA preparation, several external RNA control transcripts with varying absolute and relative abundances will be prepared as pools. Furthermore, external RNA control transcripts with different concentrations will also be spiked into a given human total RNA preparation, either as a pure RNA form or as an encapsulated form. The characteristics of such RNA pools and the total RNA preparations will be examined by RT-PCR. The following parameters will be examined.

- *Sensitivity and detection limit*: the lowest concentration of a given external RNA control transcript in the pools or in a spiked total RNA preparation will be determined by RT-PCR. The results will be compared with those obtained in its pure form.
- *Specificity*: a given external RNA control transcript in the RNA pools or in a spiked total RNA preparation should be specifically detected by RT-PCR with both 3' and 5' PCR primer sets.
- Linearity and quantification ranges: the linearity and dynamic ranges for quantitatively measuring a given external RNA control transcript in the pools or in a spiked total RNA preparation will be determined and should be comparable to those in their pure forms.
- *Precision and reproducibility*: RT-PCR reactions using multiple samples from those RNA pools and the spiked total RNA preparations will be carried out for assay precision and reproducibility. In addition, these reactions may also be performed by different operators and/or in other labs using the same assays.

6. RT-PCR protocols for QC testing

Among the scientific community two general RT-PCR methodologies are currently widely used. One-tube/one-enzyme methods utilize *Thermus thermophilus* (Tth) DNA polymerase to carry out both reverse transcriptase and DNA polymerase activities in bicine buffers containing Mn^{+2} ion. In contrast two enzyme methods typically utilize a distinct reverse transcriptase (e.g. AMV or MMLV reverse transcriptase) followed by a separate DNA polymerase to carry out the PCR amplification; the RT and subsequent PCR are usually performed in separate tubes. In general, the single-tube/single-enzyme method uses target specific primers for the reverse transcription step and subsequent amplification, whereas two-enzyme methods usually use oligo dT or random hexamer primers for the reverse transcription first strand cDNA synthesis step and target specific primers for subsequent amplification. Because of these protocol differences the two approaches may yield somewhat different results when employed with the same samples.

To simplify test procedures and reduce result comparisons, RT-PCR reagents will be purchased from a single vendor, ABI. To avoid cross contamination, a single tube SYBR Green I-based kinetic RT-PCR procedure will be employed for all QC testing (Rogge, L et al (2000) Nat. Genetics 25:96). The reagents and conditions for RT-PCR are listed below.

RT-PCR conditions using in ABI Prism® 7900HT Sequence Detection System (Applied Biosystems):

A 10 □L reaction consists of 50 mM Bicine, 125 mM KOAc, pH 8.0, 8% glycerol, 3 mM $Mn(OAc)_2$, 0.2 X SYBR□ Green I (diluted in DMSO, Molecular Probes), 0.45 □M ROX (Sigma), 200 □M dATP, 200 □M dGTP, 200 □M dCTP, 400 □M dUTP, 200 nM each primer, 0.2 units uracil N-glycosylase (Applied Biosystems), 1 unit rTth DNA polymerase (Applied Biosystems), and 2 ng total RNA or various amounts of RNA transcripts. Cycle profile: 50°C 2 min, 95°C 1 min, 60°C 30 min, followed by 95°C 30 sec, 60°C 30 sec for 50 cycles, hold at 72°C.

2.7 Comparison of external RNA controls on different technology platforms, microarray and RT-PCR

External RNA spike-in controls shall be useful on most commercially available arrays and by RT-PCR.

To assess the comparability of different RT-PCR methodologies quantitative results obtained by the SYBR-based RT-PCR protocol may be compared with results obtained using commercially available kits that AMV or MMLV reverse transcriptase and oligo dT primers coupled with target specific PCR primers and 5'nuclease probe detection approach (i.e. Taqman).

Furthermore, the results for external RNA controls and their pools obtained by RT-PCR protocols will be compared with those obtained by microarray technology. Parameters to be examined include (1) Sensitivity and detection limit, (2) Specificity, (3) Linearity of quantification range, and (4) Precision and reproducibility.

Note:

This is a topic for discussion at the workshop. We need to compare not only two different RT-PCR methodologies, but also two technology platforms. This comparison will require a broad cooperative effort.

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